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Generation of Inositol Phosphates, Cytosolic Ca^{2+}, and Ionic Fluxes in PC12 Cells Treated with Bradykinin*

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Accumulation of inositol phosphates (Ins-P_s, revealed by high performance liquid chromatography), changes of the cytosolic free Ca^{2+} ($[Ca^{2+}]_i$, revealed by fura-2), membrane potential and ionic currents (revealed by bis-oxonol and patch clamping) were investigated in PC12 cells treated with bradykinin (BK). The phenomena observed were (*a*) due to the activation of a B_2 receptor (inhibitor studies) and (*b*) unaffected by pertussis toxin, cAMP analogs, and inhibitors of either cyclooxygenase or voltage-gated Ca^{2+} channels. During the initial tens of s, three interconnected events predominated: accumulation of Ins-1,4,5-P_3, Ca^{2+} release from intracellular stores and hyperpolarization due to the opening of Ca^{2+}-activated K^+ channels. Phorbol myristate acetate partially inhibited Ins-1,4,5-P_3 accumulation at all [BK] investigated, and the $[Ca^{2+}]_i$ increase at [BK] < 50 nM. In PC12 cells treated with maximal [BK] in the Ca^{2+}-containing incubation medium, Ins-1,4,5-P_3 peaked at 10 s, dropped to 20% of the peak at 30 s, and returned to basal within 5 min; the peak increase of Ins-1,3,4-P_3 was slower and was variable from experiment to experiment, while Ins-P_4 rose for 2 min, and remained elevated for many min thereafter. Meanwhile, influx of Ca^{2+} from the extracellular medium, plasma membrane depolarization (visible without delay when hyperpolarization was blocked), and increased plasma membrane conductance were noticed. Evidence is presented that these last three events (which were partially inhibited by phorbol myristate acetate at all [BK]) were due to the activation of a cation influx, which was much more persistent than the elevation of the two Ins-P_3 isomers. Our results appear inconsistent with the possibility that in intact PC12 cells the BK-induced activation of cation influx is accounted for entirely by the increases of either Ins-1,3,4-P_3 or Ins-1,4,5-P_3 (alone or in combination with Ins-1,3,4,5-P_4), as previously suggested by microinjection studies in different cell types.

Ample evidence demonstrates that the rise of the cytosolic Ca^{2+} concentration, $[Ca^{2+}]_i$,[1] that follows the activation of receptors coupled to the hydrolysis of polyphosphoinositides (PPI) is due to at least two separate mechanisms: a transient release to the cytosol of Ca^{2+} originated from an intracellular "microsomal" store (Ca^{2+} redistribution), accompanied by a more persistent increase of the Ca^{2+} influx across the plasma membrane (1–3). The first such process is known to be mediated intracellularly by inositol 1,4,5-trisphosphate (Ins-1,4,5-P_3), a second messenger generated by the receptor-coupled reaction, whereas for the second process knowledge is still at an early stage. Indeed, studies carried out in a variety of cell types stimulated by different agonists have led to diverging proposals. In one case the influx has been suggested to be due to the opening of a true receptor-operated channel (4); in others to be triggered by intracellular second messengers such as Ca^{2+} (5), Ins-1,4,5-P_3 itself (6), Ins-1,3,4-P_3 (7, 8), or the combination of Ins-1,4,5-P_3 with its phosphorylation product, Ins-1,3,4,5-P_4 (9, 10).

In order to further characterize Ca^{2+} influx as well as the other processes brought about by the activation of PPI hydrolysis-coupled receptors, we have turned to the study of the effects of bradykinin (BK) in PC12 cells. BK is a naturally occurring nonapeptide known to elicit a large spectrum of actions. Previous studies carried out in neuroblastoma and neuroblastoma × glioma hybrid cells demonstrated the existence of BK receptors coupled to PPI hydrolysis whose activation induces $[Ca^{2+}]_i$ changes concomitant with changes of the plasma membrane potential ($\Delta\psi$), *i.e.* hyperpolarization, depolarization, or both these processes in sequence (11–17). PC12 is a line of neurosecretory cells derived from a rat pheochromocytoma, which has been extensively employed in ours as well as in other laboratories as a convenient model of nerve cells (18–21). Application of BK to PC12 cells was found to cause PPI hydrolysis, $[Ca^{2+}]_i$ rises and $\Delta\psi$ changes much larger than those induced in the same cells by the activation of another receptor, the muscarinic receptor (20, 21). The study of these various processes, in particular of their time course, in a variety of experimental conditions, led us to the conclusion that the BK-induced depolarization and Ca^{2+} influx are both due to the opening of a nonselective

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[1] The abbreviations used are: $[Ca^{2+}]_i$, free cytosolic Ca^{2+} concentration; BK, bradykinin; PPI, polyphosphoinositides; Ins-P_s, inositolphosphates; Ins-1,4,5-P_3, inositol 1,4,5-trisphosphate; Ins-(1,3,4)P_3, inositol 1,3,4-trisphosphate; Ins-(1,3,4,5)P_4, inositol 1,3,4,5-tetrakisphosphate; KRH, Krebs-Ringer medium buffered with Hepes; EGTA, [ethylenebis (oxyethylenenitrilo)]tetraacetic acid; $[Ca^{2+}]_o$, extracellular Ca^{2+} concentration; PMA, phorbol 12-myristate 13-acetate; Ins-P_4, inositol tetrakisphosphate HPLC, high performance liquid chromatography; Hepes, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid.

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influx channel, whose activation and control mechanisms we have thoroughly investigated and partially clarified.

EXPERIMENTAL PROCEDURES

PC12 cells were obtained from Dr. P. Calissano (Rome) and subcultured in our laboratory as described (22).

The standard medium (KRH) contained in mmol/liter: 125 NaCl, 5 KCl, 1 $MgSO_4$, 1 Na_2HPO_4, 5.5 glucose, 1 $CaCl_2$, and 20 Hepes (pH 7.4, 37 °C). Alternatively, $CaCl_2$ was omitted, and 1 mM EGTA was added instead. In some experiments NaCl was replaced by an equivalent amount of choline-HCl, Na_2HPO_4 by K_2HPO_4, and pH adjusted to 7.4 with Tris. The sucrose-based medium contained, in mmol/liter: 250 sucrose, 5 $KHCO_3$, 1 $MgSO_4$, 5.5 glucose, 20 Hepes, and the pH adjusted to 7.4 with Tris.

Measurement of $[Ca^{2+}]_i$—Loading with Fura-2 (23) was performed essentially as described (24). Briefly, PC12 cell suspensions (5×10^6 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 3 μM Fura-2/AM. The cells were then washed with fresh medium and left at room temperature until use. Before each experiment an aliquot of the cells was centrifuged and the pellet resuspended in the different media (see below), prewarmed at 37 °C, and supplemented with 250 μM sulfinpyrazone. The calibration of the fluorescent signal in terms of $[Ca^{2+}]_i$ was performed as described (24). Excitation and emission wavelengths were 339 and 505, respectively.

Measurement of Ca^{2+} Influx—Ca^{2+} influx was measured in two different ways: 1) by adding $CaCl_2$ to cells pre-exposed for different lengths of time to BK in Ca^{2+}-free medium, and measuring the initial rate of $[Ca^{2+}]_i$ increase, as described in Fig. 3; 2) by adding EGTA to cells prestimulated with BK in Ca^{2+}-containing medium and measuring the initial change of the rate of $[Ca^{2+}]_i$ decrease. The basic principle of this second approach, described in detail by Di Virgilio *et al.* (26) in their studies of Ca^{2+} influx through voltage-gated Ca^{2+} channels, relies on the assumption that in steady state the rate of Ca^{2+} influx is equivalent to the rate of Ca^{2+} efflux. Thus, the rate of Ca^{2+} efflux estimated after suddenly chelating $[Ca^{2+}]_o$ measures the rate of Ca^{2+} influx at the moment of EGTA addition.

Membrane Potential—The measurement of membrane potential with bis-oxonol was performed as described in detail by Di Virgilio *et al.* (26).

Patch Clamping—Measurement of plasma membrane conductance was performed in the whole cell mode of the patch clamp technique (27) as described in detail by Wanke *et al.* (28).

Inositol Phosphate Extraction and Separation—PC12 cells were incubated for 24–48 h in the RPMI-1640 inositol-free medium, supplemented with 1% fetal calf serum, and 5 μCi/ml of [^{3}H]myoinositol. The cells were then washed and resuspended in one of the saline media described above. The reaction was stopped by adding to the reaction mixtures an equivalent volume of 15% ice-cold trichloroacetic acid. The samples were incubated for 10 min on ice and then extracted 5 times with equal volumes of diethyl ether. After neutralizing with Tris, ATP (100 μM) was added and the samples applied to a Partisil Sax HPLC column (14, 29). Ins-P$_s$ were eluted with a step gradient of NH_4-formate as described in Fig. 8*a* (14, 29). 530-μl fractions were collected (flow rate 1.6 ml/min) and counted for radioactivity after mixing with 10 ml of Hionicfluor (Packard). In order to increase the recovery of Ins-P$_4$, a phytic acid hydrolysate (0.6 mg of P_i/ml of trichloroacetic acid) was included in the extraction solution (30). Such an addition caused the recovery of [^{3}H]Ins-P$_4$ standard to increase from 60 to over 85%. The identification of the different Ins-P$_s$ is based on published data (14, 29) and, for Ins-1,4,5-P$_3$ and Ins-P$_4$, on the retention time of commercial ^{3}H standards. In the experiment where the three different Ins-P$_4$ isomers were separated, the samples were extracted as described above, applied to a Partisfere 5 WAX column, and eluted by a linear gradient of $(NH_4)_2HPO_4$, pH 3.2, as described in detail by Stephens *et al.* (31). The identification of the different isomers was based on their coelution with [^{32}P]Ins-P$_4$ standards.

Materials—BK, des-Arg^9 [Leu^8]BK, [Thi^{5,8},D-Phe^7]BK, PMA, apamin, phytic acid, indomethacine, 8-Br-cAMP, and sulfinpyrazone were purchased from Sigma; Fura-2/AM, Fura-2, quin2/AM and bis-oxonol from Molecular Probes, Junction City; RPMI-1640, fetal calf serum, and horse serum from Flow Labs, Milano, Italy; inositol-free RPMI from AMIMED, Geneva, Switzerland. The B_2 specific inhibitor Arg^0 [Hyp^3,Thi^{5,8},D-Phe^7]BK was the kind gift of Dr. D. Regoli, Sherbrooke, Canada; pertussis toxin of Dr. R. Rappuoli, Siena, Italy; [^{3}H]myoinositol, [^{3}H]Ins-1,4,5-P$_3$, [^{3}H]Ins-1,3,4,5-P$_4$ were purchased from Amersham International, United Kingdom. All other materials were analytical or highest available grade.

RESULTS

Effects of BK on $[Ca^{2+}]_i$—The study of $[Ca^{2+}]_i$ by the use of Fura-2 in suspensions of intact cells is complicated by a slow leakage of the indicator to the incubation medium, which results in a progressive increase of base-line fluorescence (24, 25). Recently, DiVirgilio *et al.* (25) have demonstrated that inhibitors of organic anion transport can efficiently block the dye leakage in the macrophage cell line, J774. Using one such drug, sulfinpyrazone, which by itself has no effect on either $\Delta\psi$ or $[Ca^{2+}]_i$ in PC12 cells, no leakage of Fura-2 for up to 20 min of incubation at 37 °C, and no modification of either the size or kinetics of the $[Ca^{2+}]_i$ transients induced by various agents, including BK, could be detected. Because of this, all the $[Ca^{2+}]_i$ experiments presented here have been performed in cells pretreated with sulfinpyrazone (250 μM).

In a first series of experiments (Figs. 1 and 2), the concentration dependence of the BK effect on $[Ca^{2+}]_i$ was investigated. Concentrations of the peptide between 10^{-8}–10^{-6} M,

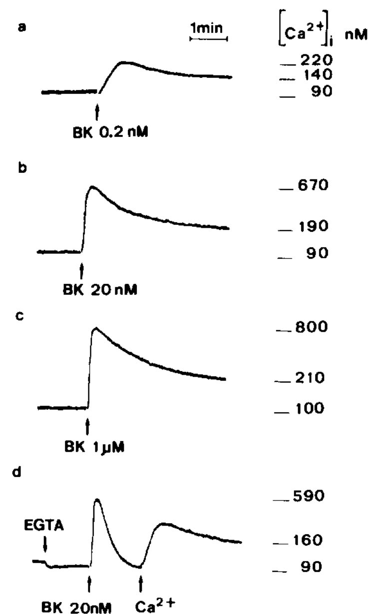

FIG. 1. **Effects of BK concentration on $[Ca^{2+}]_i$.** Conditions: Fura-2-loaded cells (see "Experimental Procedures") were incubated in Ca^{2+}-containing KRH medium. Cell number: 1×10^6/ml. In *panel d*, 1.5 mM EGTA and 1.5 mM $CaCl_2$ were added where indicated. In this and the following figures the calibrated $[Ca^{2+}]_i$ is reported on the right hand side. *Traces* shown are typical of results obtained in 10 (*a–c*) or 8 (*d*) experiments.

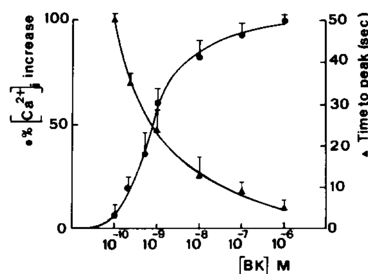

FIG. 2. **BK concentration dependence of the kinetic and amplitude of $[Ca^{2+}]_i$ rise.** Conditions as in Fig. 1. Time to peak refers to the time required to reach the maximum $[Ca^{2+}]_i$ increase. Values given are means of 10 experiments ± S.E.

administered in a complete, Ca^{2+}-containing KRH medium, caused a rapid peak increase of $[Ca^{2+}]_i$ followed by a plateau which then slowly declined toward the resting levels (Fig. 1, *b* and *c*). At [BK] below 10^{-9} M $[Ca^{2+}]_i$ transients were not only smaller, but also profoundly modified, inasmuch as the time to reach the peak increased progressively (Figs. 1*a* and 2) and no clear distinction between $[Ca^{2+}]_i$ peak and plateau was evident (Fig. 1*a*).

The results obtained in the complete KRH medium described so far were compared with those of parallel experiments, carried out by the application of BK in a Ca^{2+}-free, EGTA-containing medium (a condition in which Ca^{2+} redistribution from intracellular stores is maintained, whereas influx is no longer possible). Under these conditions the initial $[Ca^{2+}]_i$ spike was largely maintained, indicating an intracellular origin, whereas the subsequent plateau was no longer visible (compare Fig. 1, *b* and *d*), as expected for a process sustained by increased Ca^{2+} permeability at the plasma membrane. Reintroduction of Ca^{2+} into the medium bathing cells first exposed to BK in the Ca^{2+}-free medium caused $[Ca^{2+}]_i$ to rise again to levels approaching the plateau seen in the Ca^{2+}-containing medium (Fig. 1*d*), indicating that the permeability of the plasma membrane to Ca^{2+} had been increased by the peptide even at low $[Ca^{2+}]_o$. In order to establish the duration of the BK-induced Ca^{2+} influx, experiments were performed by varying the time between the addition of BK (in the Ca^{2+}-free medium) and the subsequent reintroduction of $[Ca^{2+}]_o$. As it is shown in Fig. 3*a*, an increase of Ca^{2+} influx was detected by this approach for several min after BK addition. Such an increased influx was completely dependent on BK since it was not seen in cells simply switched from a Ca^{2+}-free to a Ca^{2+}-containing medium (Fig. 3*b*). An alternative procedure employed to indirectly evaluate Ca^{2+} influx at various times after BK addition was based on the measurement of the initial rates of $[Ca^{2+}]_i$ decline observed after chelation of $[Ca^{2+}]_o$ by the addition of excess EGTA, subtracted of the decline occurring in parallel samples without EGTA addition (26). Such an approach has the advantage of being applicable even during the first min after BK, *i.e.* when redistribution of Ca^{2+} from intracellular stores predominates. With both these procedures the [BK] threshold was found to be 0.1 nM, the half-maximal effect was observed around 1.5 nM, and maximal effects above 200 nM.

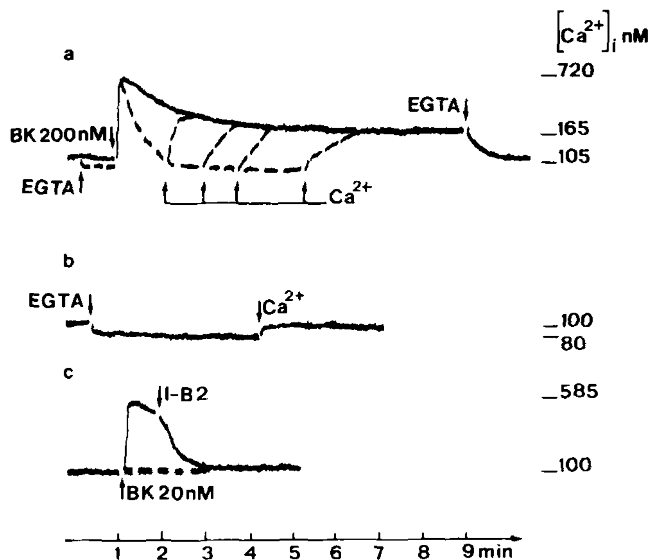

FIG. 3. **Time dependence of Ca^{2+} influx induced by BK and effects of a B$_2$ specific inhibitor on the $[Ca^{2+}]_i$ transients induced by the peptide.** *Panel a*, conditions as in Fig. 1. The continuous trace refers to cells stimulated in the presence of 1 mM CaCl$_2$ in the medium. Where indicated 1.5 mM EGTA was added. The interrupted traces refer to different aliquots from the same batch of fura-2-loaded cells stimulated with BK (200 nM) in the presence of 1.5 mM EGTA. The *arrows* refer to the time of addition of 1.5 mM CaCl$_2$ to the medium (see text for further details). *Panel b*, this trace refers to cells of the same batch as those shown in *panel a* which were not treated with BK. Where indicated 1.5 mM EGTA and 1.5 mM CaCl$_2$ were added. *Panel c*, 20 nM BK was added before (*continuous line*) or 2 min after (*interrupted line*) addition of the B$_2$ inhibitor, Arg^0 [Hyp^3,Thi^{5,8},D-Phe]BK, (I-B2, 10 μM). Typical traces from experiments repeated more than five times.

A variety of treatments was used to further characterize the $[Ca^{2+}]_i$ response of PC12 cells to BK. Cells were treated with 0.3 μM ionomycin in Ca^{2+}-free medium + 1 mM EGTA for 10 min at 37 °C. After washing with the same medium supplemented with 2% bovine serum albumin to remove the ionophore, addition of either BK or ionomycin (in Ca^{2+}-free-EGTA medium) failed to induce any significant $[Ca^{2+}]_i$ increase, indicating the depletion of the intracellular stores. In contrast, addition of BK + Ca^{2+} to cells pretreated as above resulted in a $[Ca^{2+}]_i$ increase much larger and faster than that induced by Ca^{2+} alone, indicating a stimulation of Ca^{2+} influx by the peptide. Stimulation of Ca^{2+} influx without pre-empting of the stores was demonstrated by measuring $[Ca^{2+}]_i$ in cells treated with BK after hyperloading with quin-2 to markedly increase the cytosolic Ca^{2+} buffer capacity. Under these conditions the $[Ca^{2+}]_i$ increase from intracellular stores is severely blunted (not shown in figures, Ref. 20).

Preincubation with pertussis toxin under conditions (0.1 μg/ml; 4–18 h) known to block various G$_i$ proteins (21) was without effect on both the resting $[Ca^{2+}]_i$ and on the responses induced by BK (release from intracellular stores and stimulated influx). The same was observed for the cAMP analog, 8Br-cAMP (500 μM), administered to the cells 10 min before BK (not shown in figures). Likewise, no effect was observed with the blockers of voltage-gated Ca^{2+} channels, nifedipine (up to 1 μM) and verapamil (20 μM), with a blocker of the cyclooxygenase pathway, indomethacine (10 μM), as well as with a blocker of BK receptors of the B$_1$ subtype, des-Arg^9 [Leu^8]BK (4–10 μM, not shown) (32). In contrast, the $[Ca^{2+}]_i$ increase by BK was competitively blocked by other inhibitors, [Thi^{5,8},D-Phe^7]BK and especially the more potent derivative, Arg^0[Hyp^3,Thi^{5,8},D-Phe^7]BK (100% inhibition of the effect of 2 nM BK with 1 μM inhibitor), which are specific for B$_2$ receptors (33). These B$_2$ inhibitors, when administered after BK, were also able to rapidly dissipate the plateau phase, and to bring $[Ca^{2+}]_i$ back to the resting level (Fig. 3*c*).

Another agent that induced a clear inhibition of the BK-induced $[Ca^{2+}]_i$ transients was PMA, the well-known activator of protein kinase C. The effects of PMA on BK-induced $[Ca^{2+}]_i$ redistribution and influx were investigated separately. Fig. 4 illustrates the dependence of the initial redistributive $[Ca^{2+}]_i$ peak on the concentration of both BK (*a*) and PMA (*b*) and on the length of the preincubation with the phorbol ester (*c*). As it has been described for other receptors (34–37), the inhibition required PMA concentrations in the 10^{-9} M range, and became appreciable within a few seconds after PMA addition. Different from other receptors, however, the inhibition, although marked at low peptide concentration, disappeared above 10^{-8} M BK. In order to investigate the inhibition of Ca^{2+} influx, the protocol described in Fig. 2*a* (reintroduction of Ca^{2+} into the medium bathing cells challenged with BK in Ca^{2+}-free medium 2 min earlier) was employed. Fig. 5 shows that the size of the $[Ca^{2+}]_i$ increase attained after Ca^{2+} reintroduction into the medium and, even more clearly, the rate of $[Ca^{2+}]_i$ rise were both reduced mark-

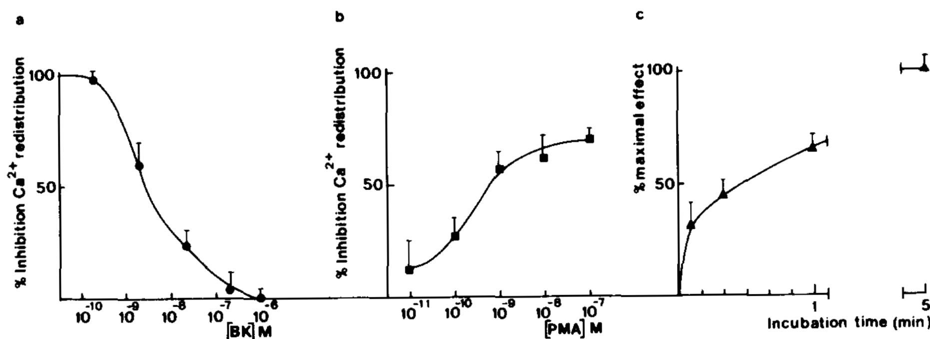

FIG. 4. **PMA inhibition of Ca^{2+} redistribution induced by BK: dependence on the concentrations of BK and of PMA, as well as on time.** Conditions as in Fig. 1, with addition of 1.5 mM EGTA 2 min before BK. In *panel a* 100 nM PMA was added 5 min before BK. In *panel b* different concentrations of PMA were added 5 min before 2 nM BK. In *panel c* 100 nM PMA was added at different times before 2 nM BK. Results in *panels a* and *b* are expressed as % inhibition compared to controls without PMA. The maximal effect of *panel c* was 60% inhibition (see *panel b*). Values given are means from four to six experiments ± S.E.

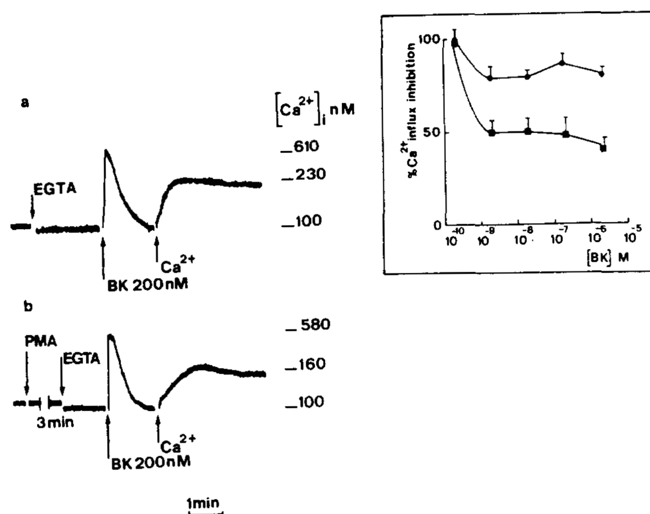

FIG. 5. **PMA inhibition of Ca^{2+} influx induced by BK.** Conditions as in Fig. 1. *Panels a* and *b*, 1.5 mM EGTA, 100 nM PMA, and 1.5 mM Ca^{2+} were added where indicated. The *inset* illustrates the dependence on [BK] of the inhibition induced by 100 nM PMA (means of four experiments ± S.E.): (■) maximal $[Ca^{2+}]_i$ rise and (●) initial rate of $[Ca^{2+}]_i$ rise measured after Ca^{2+} readdition to cells treated with BK in the Ca^{2+}-free, EGTA-containing medium (same protocol as in *panels a* and *b*).

edly by PMA. An important difference with respect to the inhibition of the redistributive peak described earlier (Fig. 4*a*) was that influx was appreciably reduced by PMA not only at low, but also at high concentrations of BK, with a degree of inhibition almost constant over a large range of peptide concentrations (10^{-9} – 10^{-6} M, *inset* in Fig. 5). The inhibition of the BK-induced $[Ca^{2+}]_i$ plateau by PMA was observed when the phorbol ester was administered not only before but also after the peptide (not shown).

A final series of experiments were carried out with PC12 cells that had been pretreated for 7–10 days with nerve growth factor (50 ng/ml). This treatment causes the cells to acquire a neuronal-like phenotype (18, 19), which is accompanied by the increased expression of some receptors, such as the muscarinic receptor (19–21). In the case of BK, however, the response in terms of $[Ca^{2+}]_i$ was found to be the same, irrespective of whether these cells had been treated or not with nerve growth factor (not shown in figures).

Effects of BK on Membrane Potential—Figs. 6 and 7 illustrate the effects of BK on $\Delta\psi$ in PC12 cells investigated under a variety of experimental conditions. These experiments were carried out by the use of the voltage-sensitive fluorescent probe, bis-oxonol. In the complete, Ca^{2+}-containing KRH the $\Delta\psi$ response to BK was found to be biphasic: hyperpolarization appeared initially, followed by a recovery to and above the resting level. A net depolarization became appreciable 30–60 s after the application of the peptide (Fig. 6*a*). Both these responses were blocked competitively by the B_2 antagonist, Arg^0 [Hyp^3,Thy^{5,8},D-Phe^7]BK. Hyperpolarization was most likely due to the transient opening of Ca^{2+}-activated K^+ channels, occurring as a consequence of the initial $[Ca^{2+}]_i$ increase. In fact, hyperpolarization was blocked by inhibitors of that type of K^+ channels, apamin and tetraethylammonium, and even better by a combination of both these drugs (38) (Fig. 6*b*), as well as by the trapping within cells of high concentrations of the fluorescent Ca^{2+} chelator, quin2 (Fig. 6*c*). In the latter condition changes of $[Ca^{2+}]_i$ that originate from limited sources, such as the intracellular microsomal store, are known to be effectively blunted (20). In contrast, the BK-induced hyperpolarization was largely preserved in the cells incubated in the Ca^{2+}-free medium (Fig. 6*d*) and was mimicked by ionomycin (Fig. 6*e*), a Ca^{2+} ionophore, used in Ca^{2+}-free, EGTA-containing medium at a concentration (2×10^{-7} M) that causes $[Ca^{2+}]_i$ rises similar in size to those induced by 10^{-7} – 10^{-6} M concentrations of BK. In cells treated first with ionomycin in the Ca^{2+}-free-EGTA medium, and then challenged with BK, the hyperpolarizing effect of the latter was no longer seen (Fig. 6*e*). This result confirms that hyperpolarization is the consequence of the redistributive $[Ca^{2+}]_i$ peak. In fact, Ca^{2+} ionophores are known to discharge intracellular Ca^{2+} pools, and thus to prevent Ca^{2+} redistribution. Finally, the effect of phorbol esters was investigated. By itself PMA had no detectable effect on $\Delta\psi$ but induced a partial inhibition of hyperpolarization at BK concentrations <10 nM (compare Fig. 6, *panel a* with *f*, and *panel g* with *h*). This result parallels the result on Ca^{2+} redistribution, illustrated in Figs. 3 and 5.

As already mentioned, an additional effect of BK on $\Delta\psi$ is a prolonged depolarization, which was of small size under standard incubation conditions (Fig. 6*a*). When, however, either blockers of Ca^{2+}-activated K^+ channels, or treatments that inhibit the redistributive $[Ca^{2+}]_i$ increase were employed, BK-induced depolarization was much greater (Fig. 6, *b*, *c*, *e*, and *l*). Under these conditions the concentration dependence of depolarization was similar to that of Ca^{2+} influx described above: threshold at 0.2 nM [BK], half-maximal effect at 2 nM,

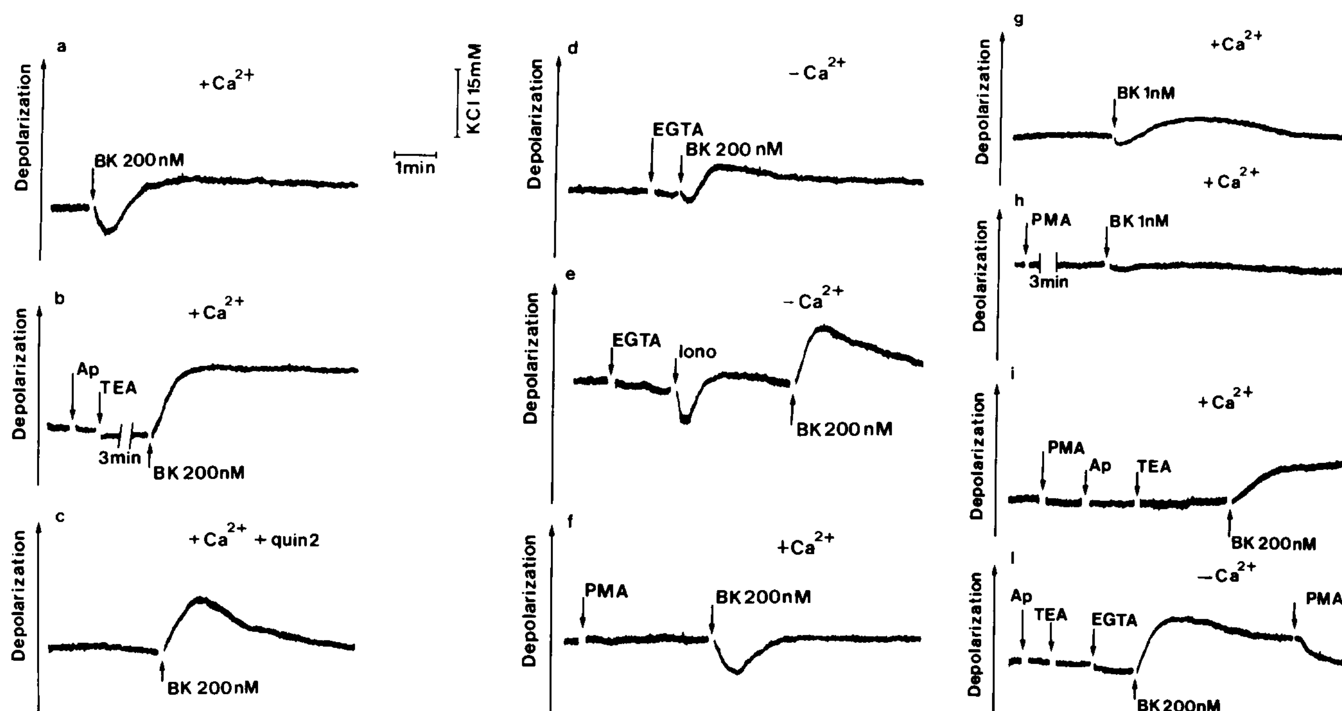

FIG. 6. **Effects of BK on membrane potential.** Cells (1×10^6/ml) in Ca^{2+}-containing KRH medium, were pre-equilibrated with 100 nM bisoxonol for 5 min at 37 °C. The traces shown are typical results of experiments that were repeated 4–20 times. 0.4 μM apamin (*Ap*), 10 mM tetraethylammonium (*TEA*), 0.2 μM ionomycin (*Iono*), 100 nM PMA, and 1.5 mM EGTA were added where indicated. Whether or not Ca^{2+} was present at the time of BK addition is indicated in each panel ($\pm Ca^{2+}$). The cells used for the experiments of *panel c* were first loaded with 30 μM quin2/AM as described in Ref. 22. The *vertical bar* on the right hand side of *panel a* represents the fluorescence increase caused by 15 mM KCl in a parallel batch of cells. This calibration refers to all the experiments presented in this figure.

maximal effect above 200 nM. The depolarization induced by the latter [BK] was similar to that of 15 mM $[K^+]_o$ (Fig. 7*a*).

When blockers of Ca^{2+}-activated K^+ channels were used, the bisoxonol signal of depolarization appeared with no appreciable lag phase after BK addition (Fig. 6, *b* and *l*), particularly when Ca^{2+}-free incubation media were used. In the latter medium depolarization was slightly reduced in size and distinctly shorter lived (6–8 instead of >10 min). As was the case with the BK-induced $[Ca^{2+}]_i$ plateau, depolarization was inhibited by PMA administered both before (compare Fig. 6, *i* and *b*) and after (Fig. 6*l*) BK. The inhibition by PMA was visible at all BK concentrations employed.

The dependence of the BK-induced depolarization on extracellular monovalent cations was investigated by the use of various Ca^{2+}-free media. Predepolarization of the cells by increased $[K^+]_o$ caused the inhibition of the BK-induced signal (Fig. 7*a* and *inset*). When NaCl of the medium was substituted with choline-HCl, depolarization was still observed (Fig. 7*b*). On the contrary, no depolarization was triggered when BK was applied in a sucrose (250 mM)-based medium (containing also $KHCO_3$, 5 mM, $MgCl_2$, 1 mM, Hepes-Tris buffer, apamin and tetraethylammonium, but neither Na^+ nor Ca^{2+}) (Fig. 7*c*). Under these experimental conditions BK was able to induce a normal Ca^{2+} redistribution from intracellular stores (not shown in figures). Na^+ or Ca^{2+} added to the sucrose-based medium caused BK-induced depolarization to reappear, although with different efficacy. In fact, as illustrated in Fig. 7 (compare *d* and *e*), the BK effect was found to be approximately 3-fold greater with 1 mM $[Ca^{2+}]_o$ than with 30 mM $[Na^+]_o$.

In a final series of experiments, the role of $[Ca^{2+}]_i$ in the BK-induced depolarization response was investigated. When cells were pretreated with ionomycin in the Ca^{2+}-free medium (under these conditions resting $[Ca^{2+}]_i$ is unaffected by BK), the depolarization induced by the peptide was still observed. In contrast, when $[Ca^{2+}]_i$ was reduced well below the resting level by loading the cells with quin2 in the Ca^{2+}-free, EGTA-containing medium (22), depolarization was markedly (50–100%) inhibited. Such an inhibition was not due to an unspecific side-effect of quin2 because, even in the cell batches where depolarization was totally abolished (four of eight experiments), reintroduction of Ca^{2+} into the medium (a treatment that causes the rapid return of $[Ca^{2+}]_i$ to the resting level, Ref. 22) resulted in the prompt resumption of the BK-induced depolarization response (not shown in figures).

Patch Clamp Studies—The bis-oxonol studies of $\Delta\psi$ were complemented by electrophysiological studies carried out in the whole-cell configuration of the patch clamp technique (27). Although performed under voltage clamp conditions previously applied with success to other cell types, such as neurons (28), these studies were difficult in PC12 cells essentially for the following two reasons: instability of membrane conductance at $\Delta\psi$ more negative than −80 mV and rapid deterioration of membrane conductance after BK application. Because of these problems, the information obtained by this approach was limited, and concerned primarily the first 1–2 min immediately following BK addition.

When the peptide (5 nM) was applied to cells internally dialyzed with a standard solution (K-aspartate, 150 mM; $[Ca^{2+}]$ buffered with EGTA at 200 nM) it induced within a few seconds the appearance of a persistent, inwardly directed current. At −45 mV $\Delta\psi$, such a current (visible in 13 of 15

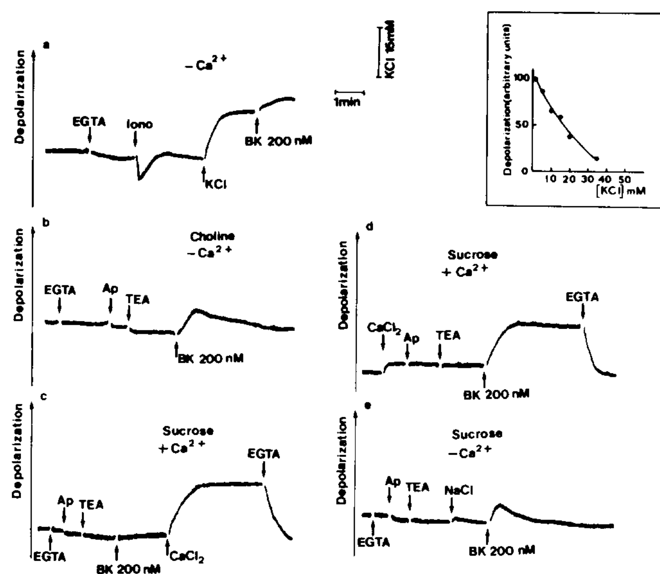

FIG. 7. **Effects of external cations on the BK-induced changes of membrane potential.** General conditions as in Fig. 6. The traces shown are typical of more than five experiments. Cells were suspended in media containing Ca^{2+}, which was chelated by excess EGTA before addition of BK ($-Ca^{2+}$), except for *panel d*, where the medium was initially without Ca^{2+}, and the latter was introduced at the beginning of the experiments ($+Ca^{2+}$). The substitutions in the medium of NaCl with either choline or sucrose are indicated in *panels b–e*. *Panel a*, KRH medium. *Panel b*, NaCl in the KRH medium was substituted with choline-HCl. *Panels c–e*, NaCl in the KRH medium was substituted with sucrose. Where indicated 1.5 mM EGTA, 0.4 μM apamin (*Ap*), 10 mM tetraethylammonium (*TEA*), 200 nM ionomycin (*Iono*), 15 mM KCl, 30 mM NaCl, 2 mM $CaCl_2$ were added. The fluorescent signal induced by 15 mM KCl is shown by the *vertical bar* to the right hand side of *panel a*. *Inset*, the cells were equilibrated in Ca^{2+}-containing KRH medium in which different concentrations of NaCl had been replaced with KCl. Two min before receiving 200 nM BK the cells were challenged with 2 mM EGTA and 200 nM ionomycin. Results (means of two consistent experiments) are expressed as % of the depolarization induced by 200 nM BK administered in a medium containing 1 mM KCl.

cells investigated) caused membrane conductance to approximately double. Moreover, in two cells an initial, short-lasting outward current was also observed. The inward current was induced by BK also at −75 mV (six of six cells). When however the $[Ca^{2+}]$ of the intracellular perfusion fluid was decreased below 10^{-9} M, the BK-induced inward current failed to appear in three of four cells and was very small and transient in the fourth.

Effects of BK on Inositolphosphate Generation—Treatment with BK in the concentration range (10^{-10} – 10^{-6} M) active on $[Ca^{2+}]_i$ and $\Delta\psi$ was found to induce a rapid accumulation of various Ins-P_s in PC12 cells, as revealed by standard HPLC analyses (29). In particular, a prompt and marked increase of Ins-1,4,5-P_3 was observed (Fig. 8). Various other Ins-P_s also increased markedly, namely an inositolmonophosphate, a bisphosphate as well as Ins-P_4 (Fig. 8). As far as Ins-1,3,4-P_3 is concerned, both the resting concentration and the percent increases with BK were quite variable, the highest the basal level, the lowest the percent increase induced by BK.

Recently, the Ins-P_4 peak separated by the standard HPLC procedure (29) was shown to contain at least three isomers, coeluting with ^{32}P standards of the Ins-D-1,3,4,6-, -D-1,3,4,5-, and -L-1,4,5,6-P_4 (31). In order to establish whether the Ins-P_4 increase noted after BK was due indeed to Ins-1,3,4,5-P_4 (*i.e.* the only isomer for which a second messenger role has been proposed (9, 10)), further studies were carried out by a new HPLC procedure, which yields a good Ins-P_4 isomer resolution (31). As can be seen in Fig. 8 (*inset*), three Ins-P_4 peaks could indeed be separated from PC12 cells by this procedure. Of these, however, only the central peak (which coelutes with the Ins-1,3,4,5-P_4 standard and which under resting conditions accounts for only a small fraction of the conventional Ins-P_4 peak) was massively (over 7-fold) increased at 20 s after BK. At 2 min Ins-1,3,4,5-P_4 was more than 10-fold basal, while the other two isomers were increased by about 50% (not shown). These results demonstrate that the changes of the Ins-P_4 isomer pattern induced by BK are complex and cannot be adequately investigated by the conventional HPLC procedure. On the other hand, the new procedure is very laborious and cannot be used routinely (31). Therefore, although the changes of the total Ins-P_4 pool were systematically measured, the contribution of the various isomers in the different experimental conditions could not be evaluated.

The time course of the accumulation of various Ins-P_s induced by 200 nM BK was investigated by the standard HPLC procedure (29) in a variety of experimental conditions. Fig. 9 illustrates results obtained with Ins-1,4,5-P_3 and Ins-P_4. The prompt initial peak increase of Ins-1,4,5-P_3 lasted less than 40 s (Fig. 9*a*) and was very much the same in the cells incubated in the complete, Ca^{2+}-containing medium, in the Ca^{2+}-free, EGTA-containing medium, and also in the cells both incubated in the latter medium and treated with ionomycin (2×10^{-7} M) 2 min before BK (not shown). In the Ca^{2+}-containing medium, Ins-1,4,5-P_3 remained slightly elevated for a few more minutes, and the resting level was reached ~5 min after BK addition (Fig. 9*a* and 10*a*), while in the Ca^{2+}-free-EGTA medium (± ionomycin pretreatment) the trend was the same, but the elevation over resting was smaller (not shown). In contrast, in the cells hyperloaded with quin2 in the Ca^{2+}-free-EGTA medium (a condition in which $[Ca^{2+}]_i$ is reduced below 15 nM, Ref. 20) the Ins-1,4,5-P_3 increase by BK was almost entirely abolished (Fig. 9*a*). As far as Ins-P_4 is concerned, the time course was distinctly different from that of Ins-1,4,5-P_3. In both the Ca^{2+}-containing and the Ca^{2+}-free-EGTA media, a linear increase of Ins-P_4 was observed during the first 20 s after BK, with a slower increment for up to 2 min (Fig. 9*b*). In the ionomycin-pretreated cells, on the other hand, the initial rise, similar to that in the other experimental conditions, was followed by a decline, which however was quite variable from experiment to experiment. Finally, in the cells loaded with quin2 and incubated in the Ca^{2+} free-EGTA-medium the Ins-P_4 increase was much smaller and slower than in the other experimental conditions (Fig. 9*b*). A large variability of the BK-induced response was observed also for Ins-1,3,4-P_3 accumulation. In some experiments the maximal increase did not exceed 50%, whereas in the group shown in Fig. 10*b* it reached 300–400%. Even in these cases the initial kinetics of Ins-1,3,4-P_3 increase was quite different from Ins-1,4,5-P_3, with the maximum reached only at 1 min, followed by a less precipitous decline (back to control at 6 min, Fig. 10*b*). Similar to $[Ca^{2+}]_i$ and $\Delta\psi$, also the BK-induced generation of Ins-1,4,5-P_3, Ins-1,3,4-P_3, and Ins-P_4 was partially inhibited by PMA (not shown).

Correlation between Inositolphosphate Generation, Ca^{2+} Influx, and Plasma Membrane Depolarization—The time course of Ins-1,4,5-P_3 accumulation in PC12 cells stimulated with 200 nM BK in the Ca^{2+}-containing medium is plotted in Fig. 10*a* together with the corresponding data on Ca^{2+} influx obtained from efflux rate measurements after EGTA addition (26). In the 2–8-min time period, similar results were obtained by the other procedure of influx measurement, *i.e.* by Ca^{2+}

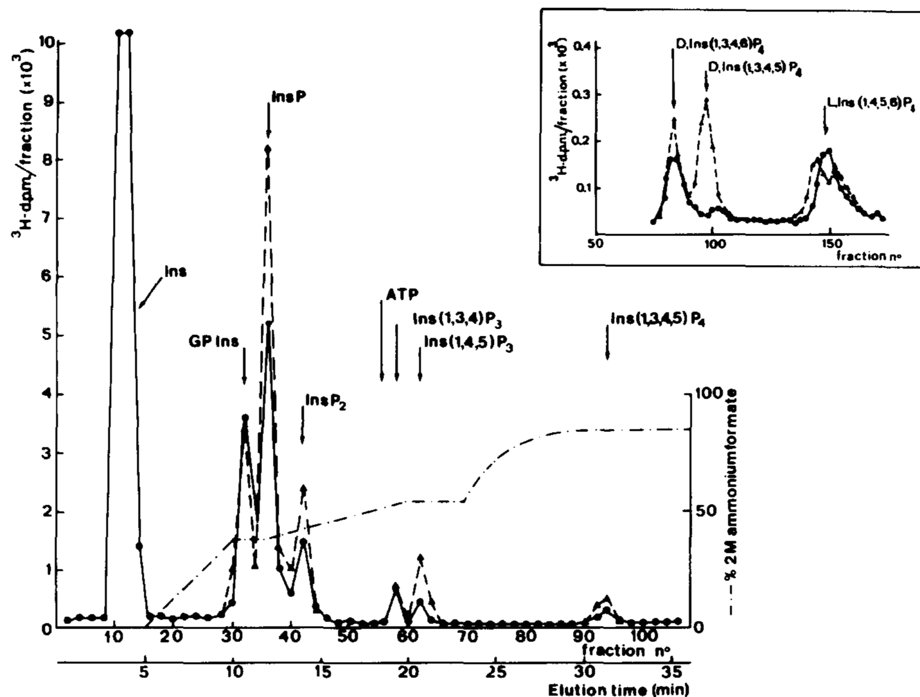

FIG. 8. **HPLC profiles of aqueous [^{3}H]inositol-labeled cell extracts.** Extracts prepared from parallel cell aliquots incubated for 10 s in Ca^{2+}-containing KRH medium either without (controls, ●) or with 200 nM BK (▲) were separated on a Partisil SAX column. *Inset*, partisphere 5 WAX HPLC profiles of [^{3}H]-Ins-P_4 isomers from controls (●) and cells stimulated with 200 nM BK for 20 s in Ca^{2+}-containing KRH medium (▲).

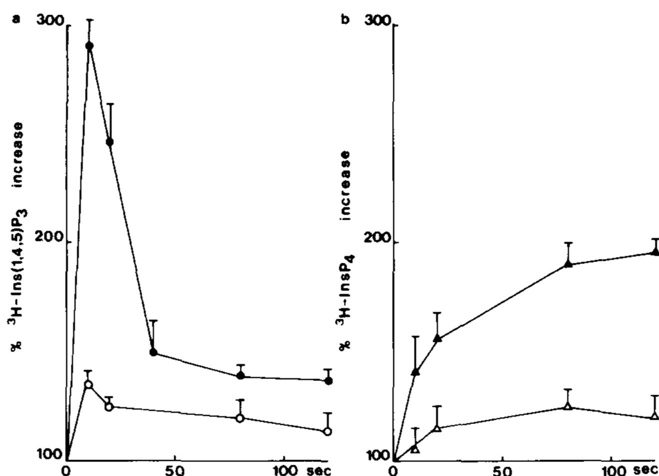

FIG. 9. **[^{3}H]Ins-(1,4,5)-P_3 and [^{3}H]Ins-P_4 changes in BK-stimulated cells.** *Panels a* and *b*, time course of [^{3}H]Ins-(1,4,5)-P_3 (● and ○, *panel a*) and [^{3}H]Ins-P_4 (▲ and △, *panel b*) increases induced by 200 nM BK. Conditions: *solid symbols*, Ca^{2+}-containing KRH medium; *open symbols*, KRH medium plus 2 mM EGTA with cells first loaded with 30 μM quin2/AM in RPMI-1640 medium supplemented with 2 mM EGTA as described in Ref. 22. Under these latter conditions $[Ca^{2+}]_i$ drops below 15 nM. Values shown are means of five experiments ± S.E., expressed as % increase compared to unstimulated samples.

reintroduction into the medium (Fig. 3*a*). As can be seen, after the initial, rapid increase, Ca^{2+} influx was dissociated from Ins-1,4,5-P_3, in particular ~40% of the maximal influx still remained after 5 min, when Ins-1,4,5-P_3 was back to resting.

A dissociation between Ins-1,3,4-P_3 and Ca^{2+} influx was also observed. In particular, the peak of Ins-1,3,4-P_3 was reached 1–2 min after BK addition, while influx was already declining at that time (not shown). An even clearer dissociation (Fig. 10) was observed in cells pretreated with ionomycin in Ca^{2+}-free + EGTA medium between the two Ins-P_3 isomers on the one hand and depolarization on the other. Under these experimental conditions the Ins-P_3 isomers reached basal values with 5–6 min (Fig. 10, *a* and *b*) when cells were still depolarized (Fig. 10*c*). This effect was not due to unspecific damage or to the slow response of bisoxonol because, when the B_2 inhibitor Arg^0 [Hyp^3,Thi^{5,8},D-Phe^7]BK was added to these ionomycin-treated cells, $\Delta\psi$ rapidly returned to resting (Fig. 10*c*). In contrast, an excellent agreement was observed in all cases between Ca^{2+} influx and plasma membrane depolarization in Ca^{2+}-containing medium. Both these effects of BK remained elevated and inhibitable by the B_2 blocker for periods longer than 8–10 min.

DISCUSSION

In the present work, a spectrum of triggered events was investigated in PC12 cells treated with the natural nonapeptide, BK, under a variety of experimental conditions. Based on their time course, the events investigated could be grouped into two classes. The first included a number of transient phenomena, *i.e.* the marked initial accumulation of Ins-1,4,5-P_3, the peak $[Ca^{2+}]_i$ increase due to redistribution from intracellular stores, and the concomitant, marked plasma membrane hyperpolarization. While these initial phenomena were subsiding, at least in part, the much more persistent phenomena of the second class became discernible or reached their peak: accumulation of various other Ins-P_s, (Ins-P_4 and Ins-1,3,4-P_3), stimulation of Ca^{2+} influx, and plasma membrane depolarization. Inhibitor studies demonstrated that the BK receptors responsible for all these phenomena were of the B_2 type (32). Similar to sensory neurons (39), but different from neuroblastoma cells (15, 17) the BK effects in PC12 cells were unaffected by pertussis toxin. They were also unaffected by 8Br-cAMP, verapamil, nifedipine, and indomethacine, indicating that neither the adenylate cyclase-cAMP system, nor the voltage-gated Ca^{2+} channels, nor the cyclooxygenase pathway were involved.

Transient Phenomena—By and large, these phenomena closely resembled those elicited in the same cells by the activation of other receptors (such as the muscarinic M_1 receptor (20, 21)), as well as the initial phenomena induced

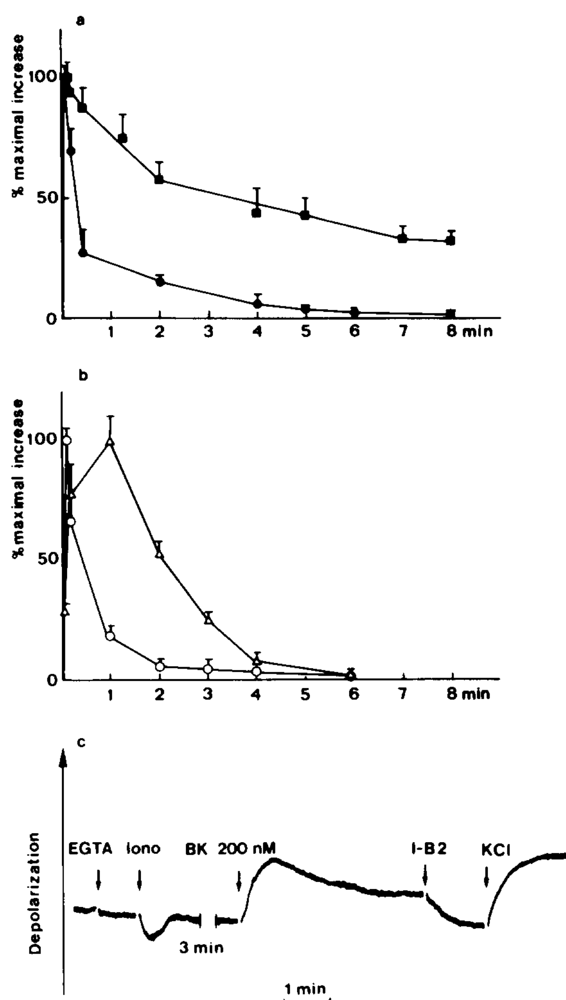

FIG. 10. **Time course of [^{3}H]Ins-(1,4,5)-P$_3$ and [^{3}H]Ins-(1,3,4)-P$_3$ production: comparison with Ca^{2+} influx and plasma membrane depolarization.** Data from *panels a* and *b* (means ± S.E. $n = 5$) come from separate experiments carried out as in Fig. 9. *Panel a*, incubations in Ca^{2+}-containing KRH medium. *a*, ●, [^{3}H]Ins-(1,4,5)-P$_3$; ■, Ca^{2+} influx, measured by the EGTA protocol in steady state described under "Experimental Procedures." *b*, incubations in Ca^{2+}-free + EGTA medium with cells pretreated with 0.3 μM ionomycin for 3 min. This panel shows the results of the five experiments in which [^{3}H]Ins-(1,3,4)-P$_3$ (△) increases were the largest (peaks = 300–400% of basal). ○, [^{3}H]Ins-(1,4,5)-P$_3$. *Panel c* illustrates the effects of BK and of a B$_2$ antagonist on $\Delta\psi$, under the incubation conditions used for *panel b* and Fig. 6*e*. Where indicated 1.5 mM EGTA, 200 nM ionomycin (*Iono*), 30 μM Arg^0, [Hyp^3,Thi^{5,8},D-Phe^7] BK (*I-B2*), and 15 mM KCl were added.

by BK in other cell types (11–17, 39). A large body of literature indicates that these transient phenomena are not independent from each other, but rather causally related. Thus, Ins-1,4,5-P$_3$, a primary metabolite of PIP$_2$ hydrolysis, is known to cause release of Ca^{2+} from a microsomal intracellular store (1, 2). The ensuing increase of $[Ca^{2+}]_i$, on the other hand, can trigger the opening of Ca^{2+}-activated K^+ channels. These channels, which are known to be largely expressed in PC12 cells (38), are in turn responsible for the hyperpolarization response, as demonstrated by *a*) the blocking effect of inhibitors, apamin and tetraethylammonium, and (*b*) the disappearance of hyperpolarization in cells either pretreated with ionomycin in a Ca^{2+}-free medium, or heavily loaded with the fluorescent Ca^{2+} chelator, quin2. The first treatment mentioned in *b* causes the discharge of intracellular Ca^{2+} stores, and therefore prevents the receptor-triggered Ca^{2+} redistribution; the second markedly increases the cytosolic Ca^{2+} buffering capacity (20, 22).

As it has been reported for a number of other receptors coupled to PPI hydrolysis (34–37), the generation of Ins-1,4,5-P$_3$ at the B$_2$ receptors of BK was inhibited by PMA, presumably through the activation of the enzyme target of this drug, protein kinase C. It may therefore be hypothesized that in PC12 cells B$_2$ receptors operate under a negative auto-feedback control mediated by diacylglycerol, the physyological activator of protein kinase C and another second messenger generated by PPI. Under the same experimental conditions, inhibition by PMA of Ca^{2+} redistribution was also observed, but only at submaximal BK concentrations. This nonlinear relationship between Ins-1,4,5-P$_3$ generation and Ca^{2+} redistribution will be discussed in detail elsewhere.[2]

Activation of a Channel May Account for Both Ca^{2+} Influx and Plasma Membrane Depolarization—Compared with the transient phenomena, the more persistent phenomena triggered by the activation of receptors coupled to PPI hydrolysis have received, up to now, much less attention. Stimulation of Ca^{2+} influx unaffected by the usual blockers of voltage-gated Ca^{2+} channels was reported in a large number of studies (review Refs. 1–3), yet the nature of the process (involvement of either a channel or a transporter) was investigated only in a few, and a variety of control mechanisms were proposed (4–10). As far as BK-induced depolarization is concerned, work in neuroblastoma (but not in other cells, such as sensory neurons (39)) demonstrated the predominant involvement of an outward K^+ current, the M current, which appeared to be inhibited by diacylglycerol generated by receptor-triggered PPI hydrolysis (13, 15, 16). In addition, in the same neuroblastoma cells an inward cationic current stimulated by BK was described, but its nature and physiological relevance were not established (13).

In the present work, information on the persistent effects of BK in PC12 cells was obtained by the parallel study of $[Ca^{2+}]_i$, $\Delta\psi$ and ionic currents, investigated by Fura-2, bis-oxonol, and patch clamping, respectively. The whole-cell electrophysiological studies (carried out under conditions in which $[Ca^{2+}]_i$ is clamped at 10^{-7} M or less, in order to prevent activation of Ca^{2+}-dependent K^+ channels) demonstrated that BK triggers a persistent inward current, with doubling of the plasma membrane conductance. This result excludes a main role for the M current (whose attenuation by BK would be expected to cause a decrease and not an increase of membrane conductance) and indicates in contrast that activation of an inward cationic current is taking place. Because of the problems encountered in PC12 patch clamping, the channel responsible for such a current was not characterized in detail by electrophysiology. The bis-oxonol results, particularly those with sucrose medium, demonstrated however that this BK-activated channel is permeable to Ca^{2+} with some preference over Na^+. Moreover, when BK was applied in a divalent cation-free medium, even choline became able to sustain depolarization. These features suggest the involvement of a cation channel of low specificity that might be similar to the channel opened by ATP in smooth muscle cells (4).

Since this BK-activated cation channel appears permeable to Ca^{2+}, it was a logical candidate to account for the Ca^{2+} influx elicited by the peptide. This conclusion is supported also by the results of other experiments in which Ca^{2+} influx and depolarization were found to remain associated in a variety of conditions. In fact, both these processes were found to be persistent, they exhibited very similar concentration dependence for BK, and were both inhibited by PMA over

[2] C. Fasolato and T. Pozzan, manuscript in preparation.

the entire range of BK concentrations, while Ca^{2+} redistribution was inhibited at low BK only (see above). On the other hand, Ca^{2+} antagonists, nifedipine and verapamil, failed to modify the effects of BK. L-type channels, which are the targets of these drugs and the voltage-gated Ca^{2+} channels expressed in undifferentiated PC12 cells (26, 40), appear therefore to play no major role in the spectrum of action of BK, presumably because depolarization by the peptide remains subthreshold for their activation. The recognition that Ca^{2+} influx and depolarization induced by BK in PC12 cells may be due to one and the same process was particularly important. In fact, at variance with Ca^{2+} influx studies, that require reasonable $[Ca^{2+}]_o$ to be carried out, $\Delta\psi$ studies could be extended to other experimental conditions, including the use of various Ca^{2+}-free media as well as the large decrease of $[Ca^{2+}]_i$ and discharge of intracellular Ca^{2+} stores, obtained by loading the cells with quin2 and by treatment with ionomycin during incubation in such media, respectively.

The BK-induced Ca^{2+} influx and depolarization were not stimulated but rather inhibited by PMA. This result is again at variance with those in neuroblastoma cells, where PMA was found to inhibit the M current, and thus to cause depolarization (13, 16, 17). We conclude that in PC12 cells the endogenous PMA analog and protein kinase C activator, diacylglycerol, cannot be the trigger of the BK-activated channel but in contrast could act as a negative modulator. Both Ca^{2+} influx stimulated by BK applied together with Ca^{2+} reintroduction into the medium, and depolarization induced by the peptide in Ca^{2+}-free media were still visible after discharge of intracellular Ca^{2+} stores (by ionomycin) and increase of $[Ca^{2+}]_i$ buffer (by high quin2). Since in both these conditions $[Ca^{2+}]_i$ is clamped near the resting level (10^{-7} M), our results exclude that in PC12 cells the trigger of the BK-activated channel is $[Ca^{2+}]_i$ increase above resting, as it was suggested in granulocytes activated with the chemotactic peptide, fMLP (5). $[Ca^{2+}]_i$, however, appears to have some permissive role in channel opening. In fact, when $[Ca^{2+}]_i$ was decreased to very low levels ($10^{-8} - 10^{-9}$ M) both depolarization revealed by bis-oxonol, and the inward current revealed by patch clamping were inhibited to a large extent.

Role of Ins-P$_s$ in Channel Operation—In previous studies, microinjection within various cell types of different Ins-P$_s$ (in particular, Ins-1,3,4-P$_3$, Ins-1,4,5-P$_3$, and the combination of the latter with Ins-1,3,4,5-P$_4$) was reported to induce the activation of cation influx (6–10). Based on these data, the receptor-triggered generation of these various Ins-P metabolites was suggested to be responsible for the activation of the cation influx also in intact cells. From the present studies of the BK effects in PC12 cells, a causal role of Ins-1,3,4-P$_3$ in the influx stimulation appears unlikely because the increase of this metabolite was quite variable from experiment to experiment, whereas Ca^{2+} influx and depolarization were not, and its kinetics dissociated from that of Ca^{2+} influx and depolarization. Ins-1,4,5-P$_3$, on the other hand, increased rapidly after BK addition, but then returned to the base-line level much earlier than Ca^{2+} influx and depolarization. Because of the complexity of the Ins-P$_4$ isomer spectrum, and the technical problems in the separation of the various components, a detailed analysis of the Ins-1,3,4,5-P$_4$ time course after BK could not be carried out. This metabolite was found to increase rapidly, but its persistence during BK treatment remains undefined. In spite of this limitation, our results provide new information in this field. In fact, microinjected Ins-1,3,4,5-P$_4$ always required the presence of Ins-1,4,5-P$_3$ for activity (9, 10). The results obtained with the combination of the two Ins-P$_s$ were embodied into a model, where Ins-1,4,5-P$_3$ and Ins-1,3,4,5-P$_4$ act in tandem, the latter by activating a Ca^{2+} pathway directly from the extracellular space to the microsomal store (possibly located in close apposition to the plasma membrane), the former by causing Ca^{2+} discharge from the store to the cytosol (4, 41). Because of the relatively rapid return of Ins-1,4,5-P$_3$ to the resting level, our present results in intact PC12 cells appear incompatible with this model. Thus, Ins-1,4,5-P$_3$ could, at the best, only prime the channel to a persistent activation by Ins-1,3,4,5-P$_4$. Alternatively, Ins-1,4,5-P$_3$ and Ins-1,3,4,5-P$_4$ could be responsible for only a part of influx regulation, for example they could work in the initiation and/or positive modulation of a response sustained by the direct receptor-channel interaction in the plane of the membrane. This type of interaction has been reapeatedly suggested to account for the modulation of voltage gated K^+ and Ca^{2+} channels (42, 43). The alternative model is appealing also because it combines in one single proposal two mechanisms that were envisaged separately. At the present time, however, no specific supportive evidence is available, and the model can therefore be regarded only as an initial guideline for future work in the field.

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REFERENCES

1. Berridge, M. J. (1987) *Annu. Rev. Biochem.* **56,** 159–193
2. Putney, J. W. (1987) *Trends Pharmacol. Sci.* **8,** 481–486
3. Meldolesi, J., and Pozzan, T. (1987) *Exp. Cell Res.* **171,** 271–283
4. Benham, C. D., and Tsien, R. W. (1987) *Nature* **328,** 275–278
5. Tscharner, V., Prod'hom, B., Baggiolini, M., and Reuter, H. (1986) *Nature* **324,** 369–372
6. Kuno, M., and Gardner, P. (1987) *Nature* **326,** 301–303
7. Tertoolen, L. G. J., Tilly, B. C., Irvine, R. F., and Moolenaar, W. H. (1987) *FEBS Lett.* **214,** 365–369
8. Higashida, H., and Brown, D. A. (1986) *FEBS Lett.* **208,** 283–286
9. Irvine, R. F., and Moor, R. M. (1986) *Biochem. J.* **240,** 917–920
10. Morris, A. P., Gallacher, D. V., Irvine, R. F., and Petersen, O. H. (1987) *Nature* **330,** 653–656
11. Reiser, G., and Hamprecht, B. (1985) *Pfleuger's Arch.* **405,** 260–264
12. Yano, K., Higashida, H., Hattori, H., and Nozawa, Y. (1985) *FEBS Lett.* **181,** 403–405
13. Higashida, H., Streaty, R. A., Klee, W., and Nirenberg, M. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 942–946
14. Jackson, J. R., Hallam, T. J., Downes, C. P., and Hanley, M. R. (1987) *EMBO J.* **6,** 49–54
15. Francel, P. C., Miller, R. J., and Dawson, G. (1987) *J. Neurochem.* **48,** 1632–1639
16. Miller, R. J. (1987) *Trends Neurosci.* **10,** 226–228
17. Brown, D. A., and Higashida, H. (1988) *J. Physiol.* **397,** 185–207
18. Greene, L. A., and Tischler, A. (1976) *Proc. Natl. Acad. Sci. U. S. A.* **73,** 2424–2428
19. Greene, L. A. (1984) *Trends Neurosci.* **9,** 91–94
20. Pozzan, T., DiVirgilio, F., Vicentini, L. M., and Meldolesi, J. (1986) *Biochem. J.* **234,** 547–553
21. Vicentini, L. M., DiVirgilio, F., Ambrosini, A., Meldolesi, J., and Pozzan, T. (1986) *Biochem. J.* **234,** 555–562
22. Pozzan, T., Gatti, G., Dozio, N., Vicentini, L. M., and Meldolesi, J. (1984) *J. Cell Biol.* **99,** 628–638
23. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) *J. Biol. Chem.* **260,** 3440–3450
24. Malgaroli, A., Milani, D., Meldolesi, J., and Pozzan, T. (1987) *J. Cell Biol.* **105,** 2145–2153
25. DiVirgilio, F., Steinberg, T. H., Swanson, J. A., and Silverstein, S. C. (1988) *J. Immunol.* **140,** 915–920
26. DiVirgilio, F., Milani, D., Leon, A., Meldolesi, J., and Pozzan, T. (1987) *J. Biol. Chem.* **262,** 9189–9195
27. Hamill, O. P., Marty, A., Neher, E., Sakmann, B., and Sigworth, F. J. (1981) *Pflueger's Arch.* **391,** 85–100

28. Wanke, E., Ferroni, A., Malgaroli, A., Ambrosini, A., Pozzan, T., and Meldolesi, J. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 4313–4317
29. Batty, I. R., Nahorski, S. R., and Irvine, R. F. (1985) *Biochem. J.* **232,** 933–934
30. Wregget, K. A., Howe, L. R., Moore, J. P., and Irvine, R. F. (1987) *Biochem. J.* **245,** 933–934
31. Stephens, L. R., Hawkins, P. T., Barker, C. J., and Downes, C. P. (1988) *Biochem. J.* **253,** 721–733
32. Regoli, D., and Barabé, J. (1980) *Pharmacol. Rev.* **32,** 1–42
33. Regoli, D., Rhaleb, N. E., Drapeau, G., Dion, S., Tousignant, C., and D'Orleans-Juste, P. (1988) *Kinins V,* Plenum Publishing Corp., New York, in press
34. Vincentini, L. M., DiVirgilio, F., Ambrosini, A., Pozzan, T., and Meldolesi, J. (1985) *Biochim. Biophys. Res. Commun.* **127,** 310–317
35. Watson, S. P., and Lapetina, E. G. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 2623–2626
36. Bouvier, M., Leeb-Lundberg, L. M. F., Benovic, J. L., Caron, M. G., and Lefkowitz, R. J. (1987) *J. Biol. Chem.* **262,** 3106–3113
37. Nishizuka, Y. (1986) *Science* **233,** 305–31
38. Schmid-Antomarchi, H., Hugues, M., and Lazdunski, M. (1986) *J. Biol. Chem.* **261,** 8633–8637
39. Thyer, S. A., Ewald, D. A. Perney, T. M., and Miller, R. J. (1988) *J. Neurosci.,* in press
40. Kongsamut, S., and Miller, R. J. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 2243–2247
41. Putney, J. W. (1986) *Cell Calcium* **7,** 1–12
42. Codina, J., Yatani, A., Grenet, D., Brown, A. M., and Birnbaumer, L. (1987) *Science* **236,** 442–445
43. Brown, A. M., and Birnbaumer, L. (1988) *Am. J. Physiol.* **254,** H401–H410